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p53 Oligomerization Is Essential for Its C-terminal Lysine Acetylation*

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Acetylation of multiple lysine residues in the p53 plays critical roles in the protein stability and transcriptional activity of p53. To better understand how p53 acetylation is regulated, we generated a number of p53 mutants and examined acetylation of each mutant in transfected cells. We found that p53 mutants that are defective in tetramer formation are also defective in C-terminal lysine residue acetylation. Consistently, we found that several cancer-derived p53 mutants that bear mutations in the tetramerization domain cannot form oligomers and are defective in C-terminal lysine acetylation, and these mutants are inactive in p21 transactivation. We demonstrated that the acetyltransferase p300 interacts with and promotes acetylation of wild-type p53 but not with any of the artificially generated or human cancer-derived p53 mutants that are defective in oligomerization. These results, combined with a computer-aided crystal structure analysis, suggest a model in which p53 oligomerization precedes its acetylation by providing docking sites for acetyltransferases.

The tumor suppressor p53 is a critical mediator of the cellular stress response, maintaining genomic integrity and preventing oncogenic transformation by inducing both cell cycle arrest and apoptotic cell death (1). Post-translational modifications play critical roles in regulating p53 function by modulating protein stability, target gene preferences, and subcellular localization of p53. p53 is acetylated by p300/CBP,[3] a protein that possesses histone acetyltransferase activity and is a co-activator of p53 able to augment p53 transcriptional activity (2–4). p53 acetylation occurs at multiple lysine residues in the C terminus of p53 (residues 370, 372, 373, 381, and 382) in response to DNA-damaging agents (5–7). Acetylation of these lysine residues stabilizes p53 protein (8), enhances its sequence-specific DNA binding (9), and augments p53 recruitment of transcriptional activators (10). Intriguingly, these C-terminal acetylation sites of p53 are also essential for MDM2-induced ubiquitination and degradation (11). DNA damage-induced p53 acetylation can be inhibited by MDM2 and reversed by co-expression of the tumor suppressor ARF (alternative reading frame) (8), implying a connection between ARF function and p53 acetylation. Furthermore, acetylation of p53 in the DNA binding core domain has recently been shown to play a role in determining whether p53 induces cell cycle arrest or apoptosis (12, 13). Nevertheless, the spatial and temporal regulation of p53 acetylation is not fully understood, and the relationship between p53 oligomerization and acetylation has not been studied.

It is believed that p53 exists predominantly in a monomeric state when expressed at low levels under unstressed conditions (14–16), and the protein functions most efficiently as a tetramer because of the high affinity of the tetramer for binding DNA (17). Tetramerization of p53 is a function of the C-terminal domain, spanning residues 325–356, which can by itself form tetramers in solution (18). Each tetramer is a symmetric dimer of primary dimers in which all four subunits are geometrically equivalent (18, 19) (also see Fig. 7*B*). The tetramerization domain consists of a short β-strand and an α-helix that are connected via a sharp hairpin. Through intermolecular β-sheet formation and helix packing, four monomers assemble to a tightly packed tetramer, which can efficiently bind to DNA (18, 19). Interestingly, the C-terminal tetramerization domain of p53 harbors a nuclear export signal (NES) sequence, spanning residues 340–351, that can be masked by formation of tetramers, indicating that regulation of p53 tetramerization and nuclear export are interlinked (20). Mutations in the p53 tetramerization domain have been found in human cancers, although less frequently than mutations occurring in the DNA binding domain. Intriguingly, it appears that mutations in the tetramerization domain are more frequently associated with p53 germ line mutations. For example, mutations on Arg-337 within the tetramerization domain account for about 20% of the reported cases of germ line p53 mutations found in patients with Li-Fraumeni syndrome (21). As such, Arg-337 is much more frequently mutated in Li-Fraumeni syndrome patients than the other cancer hot spot sites in the DNA binding domain. Another cancer-associated p53 mutation within the tetramerization domain, L344P, is also believed to be the causative germ line mutation in an identified Li-Fraumeni syndrome family (22). Despite these observations, the basis for the

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[3] The abbreviations used are: CBP, CREB-binding protein; GFP, green fluorescent protein; TSA, trichostatin A; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; NLS, nuclear localization signal; NES, nuclear export signal; LMB, leptomycin B; pKI, protein kinase inhibitor.

preferential occurrence of mutations in the p53 tetramerization domain in the germ line remains unclear.

In this study, we have explored the regulation of p53 acetylation using a series of p53 tetramerization domain mutants. Our data indicate that the p53 C-terminal lysine acetylation occurs much more efficiently on p53 tetramers, less so on p53 dimers, and almost cannot occur on p53 monomers. Our data also indicate that the acetyltransferase p300 interacts with and promotes acetylation of wild-type p53, but not with any of the artificially generated or human cancer derived p53 mutants that are defective in oligomerization. Based on our findings, we propose a model in which DNA damage leads to formation of p53 tetramers; acetyltransferases, such as p300/CBP, then associate with and acetylate the C-terminal lysines on p53 tetramers. Acetylation of p53 C-terminal lysine residues simultaneously prevents Mdm2-induced ubiquitination on the same lysine residues and further stabilizes the tetramer, facilitating its sequence-specific DNA binding as well as recruitment of transcriptional co-activators.

EXPERIMENTAL PROCEDURES

Plasmids—The construction of full-length human p53 plasmids was described previously (23). p53 mutations were introduced by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) and verified by DNA sequencing. p53 constructs fused with GFP at the C terminus were used in the experiments for monitoring the localization of p53 in living cells.

Cell Culture and Chemical Treatments—Human osteosarcoma U2OS cells and human lung tumor H1299 cells were purchased from ATCC. Cells were routinely maintained in a 37 °C incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. In all cases, cells were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Where indicated, cells were treated with 0.5 μM histone deacetylase inhibitor trichostatin A (TSA) (Sigma) and 5 mM nicotinamide (Sigma) for 6 h.

Western Blot Analysis—Cells were transiently transfected with plasmids for 24 h prior to cell lysis. GFP plasmid was co-transfected as a control for transfection efficiency. Cells were lysed in 2% SDS lysis buffer (2% (w/v) SDS, 50 mM Tris-HCl (pH 6.8), 10% glycerol) after transfection. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat dried milk in phosphate-buffered saline containing 0.1% (v/v) Tween 20 (PBST) and incubated with primary antibodies and horseradish peroxidase-labeled secondary antibodies diluted in the blocking buffer. The blots were thoroughly rinsed with PBST before and after incubations, and the signals were detected with chemiluminescence detection reagents according to the manufacturer's instructions (Pierce). Antibodies for p53 (NeoMarker, DO-1; Santa Cruz Biotechnology, FL393), acetylated p53 (Lys-382) (Cell Signaling), actin (Chemicon), and GFP (Research Diagnostics) were commercially purchased. Rabbit anti-p21 antibody was a gift from Dr. Yue Xiong (University of North Carolina, Chapel Hill). To detect the acetylation at p53 C terminus without using TSA and nicotinamide (only in Fig. 1*C*), cells were lysed in 1% SDS lysis buffer (1% SDS and 1% Nonidet P-40 in PBS). The cell lysates were diluted 10 times with 0.1% Nonidet P-40/PBS containing 1 mM PMSF. The diluted lysates were pre-cleaned with Sepharose CL4B beads (Sigma) for 30 min and then immunoprecipitated with goat anti-p53 antibody (FL393) overnight at 4 °C, followed by incubation with protein A/G beads (Pierce) for 2 h at 4 °C. The beads were washed four times with cold 0.1% Nonidet P-40/PBS containing 1 mM PMSF. The beads were then incubated in 1× loading buffer at 100 °C for 5 min, followed by SDS-PAGE. The level of p53 acetylation was determined with rabbit anti-pan-Ace-p53 antibody (a gift from Dr. Wei Gu, Columbia University).

Protein Cross-linking Assay—Cells were transfected with the indicated plasmids and lysed with 0.5% Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM $NaVO_3$, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture) 36 h after transfection. Glutaraldehyde was added to the lysate at indicated concentrations. After incubating the lysate on ice for 20 min, the glutaraldehyde reactions were stopped by adding 2× loading buffer, and the samples were heated at 100 °C for 5 min and resolved by SDS-PAGE. Western blot analysis was performed with anti-p53 antibody (DO-1).

Immunoprecipitation—Cells were transfected with the indicated plasmids for 36 h and were cross-linked with 5 mM dimethyl 3,3′-dithiobispropionimidate 2HCl in PBS for 1 h at room temperature. The cells were then lysed with 0.5% Nonidet P-40 lysis buffer. The lysates were pre-cleaned with Sepharose CL4B beads (Sigma) for 30 min, and immunoprecipitated with rabbit anti-HA antibody (Santa Cruz Biotechnology, Y11) overnight at 4 °C, followed by incubation with protein A/G beads (Pierce) for 2 h at 4 °C. The beads were washed four times with cold 0.5% Nonidet P-40 lysis buffer. Following the immunoprecipitation, cross-links were reversed prior to loading by boiling the beads in 1× loading buffer at 100 °C for 5 min, and then samples were resolved by SDS-PAGE.

RESULTS

Nuclear-confined p53 NES Mutant Is Defective in Lys-382 Acetylation—p53 shuttles between the nucleus and cytoplasm via its bipartite nuclear localization signal (NLS) (24) and its nuclear export signal (NES) (20, 25) sequences. To determine the spatial regulation of p53 acetylation, we constructed $p53^{NLS}$ and $p53^{NES}$ mutants (Fig. 1*A*) that were deficient in nuclear import or nuclear export activities (Fig. 1*B*) (23). We expressed the p53 mutants in non-small cell lung cancer H1299 (p53-negative) tumor cells. One day after transfection, cell lysates were collected, and the amount of constitutive p53 acetylation was determined using a polyclonal antibody specific to Lys-382-acetylated p53. We carried out most of our analysis using anti-Lys-382 acetylation antibody, because previous studies have shown that Lys-382 is the most highly acetylated site in the p53 protein (5) and that antibodies to Lys-382-acetylated and pan-acetylated (lysines 370, 372, 373, 381 and 382) p53 yield very similar results (8) (also see Fig. 5*C*). Wild-type p53 and the cytoplasmic-localized $p53^{NLS}$ mutant were acetylated at Lys-382 with comparable efficiency (Fig. 1*C*, *lanes 2* and *3*). In contrast, the $p53^{NES}$ mutant, despite its wild-type-like nuclear

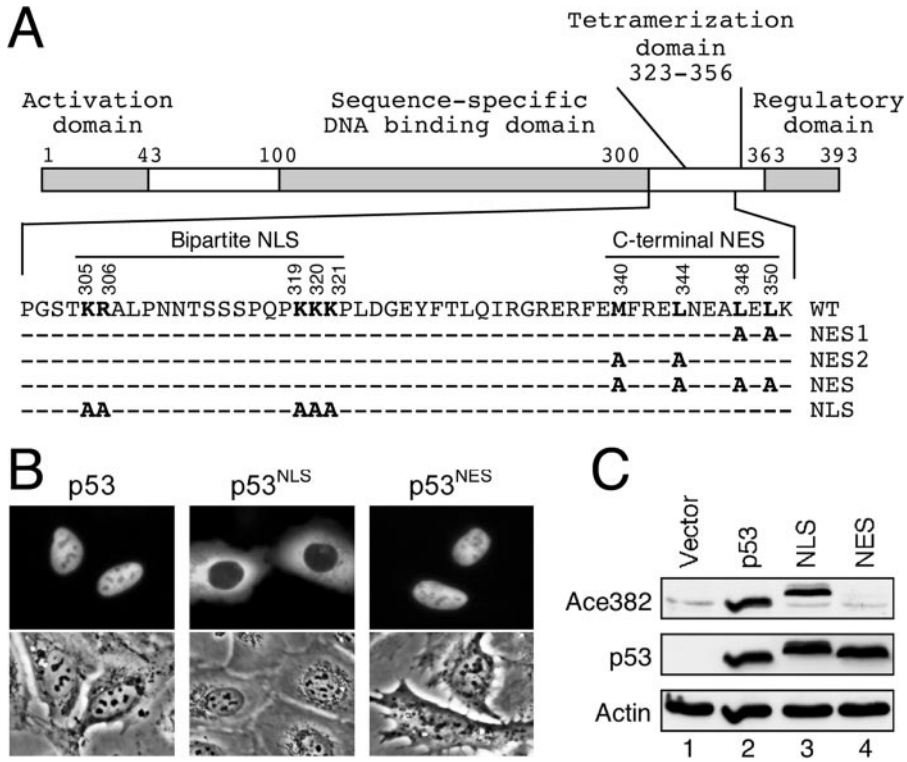

FIGURE 1. **Nucleus-confined but not cytoplasm-confined p53 is defective in Lys-382 acetylation.** *A,* diagram of human p53 protein with amino acid sequences of the bipartite NLS and the C-terminal NES indicated. The functionally important basic amino acid residues in the NLS and hydrophobic amino acid residues in the NES are shown in *boldface* in the wild-type (*WT*) sequence. Alanine substitutions in the p53 mutants are indicated. *B,* localization of wild-type and mutant p53 proteins. U2OS cells were transiently transfected with plasmid DNA expressing indicated p53 proteins. Fluorescence and phase contrast images were taken 24 h after transfection. *C,* detection of p53 acetylation at Lys-382. H1299 cells were transiently transfected with indicated p53 plasmid DNA. Cell lysates were collected 24 h after transfection, immunoprecipitated by anti-p53 antibody, and resolved on SDS-polyacrylamide gel and blotted with antibodies specific to Lys-382 acetylated p53 (Ace382), p53, and actin.

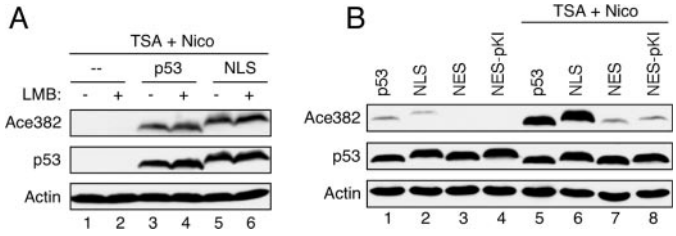

FIGURE 2. **Restoration of nuclear export function of p53^{NES} mutant cannot restore Lys-382 acetylation.** *A,* acetylation of nuclear confined p53. H1299 cells were transiently transfected with indicated p53 plasmids. One day after transfection, the cells were treated with 0.5 μM TSA plus 5 mM nicotinamide (*Nico*) in the absence (*lanes 1, 3,* and *5*) or presence (*lanes 2, 4,* and *6*) of 10 nM LMB for 1 day before lysis. p53 acetylation was analyzed by Western blotting using antibody to Lys-382 acetylated p53. *B,* restoration of nuclear export function of p53^{NES} mutant cannot restore Lys-382 acetylation. H1299 cells were transiently transfected with indicated p53 plasmids. The cells were then treated without (*lanes 1–4*) or with (*lanes 5–8*) 0.5 μM TSA plus 5 mM nicotinamide (*Nico*) for 6 h before lysis. p53 acetylation was analyzed by Western blotting using antibody to Lys-382 acetylated p53.

localization (Fig. 1*B*), was not acetylated (Fig. 1*C*, *lane 4*). This result suggests that the acetylation of p53 can occur in the cytoplasm, so that both the wild-type p53 (predominantly localizes in the nucleus and shuttles between the nucleus and the cytoplasm) and the cytoplasm-confined p53^{NLS} mutant can be acetylated, whereas the nucleus-confined p53^{NES} mutant cannot. Alternatively, the result can be explained as that the mutations introduced into the p53^{NES} mutant convey the protein resistance to acetylation by as-yet unknown mechanisms.

Restoration of Nuclear Export of p53^{NES} Mutant Cannot Restore Lys-382 Acetylation—To distinguish between these, we first determined whether a nucleus-confined wild-type p53 could be acetylated. We expressed wild-type p53 and p53^{NLS} mutant in H1299 cells and treated the cells with leptomycin B (LMB) to block p53 nuclear export and determined p53 acetylation by anti-Lys-382 acetylation antibody. Interestingly, our data showed that both the wild-type p53, which is confined in the nucleus by LMB (25), and the p53^{NLS} mutant, which is insensitive to LMB treatment and remains in the cytoplasm (23), could be acetylated at Lys-382 with similar efficiency (Fig. 2*A*), suggesting that acetylation of p53 can occur in both the nucleus and the cytoplasm. The data also suggest that the lack of Lys-382 acetylation of the p53^{NES} mutant is not because of its lacking nuclear export activity. To verify this notion, we employed a strategy to restore nuclear export function of the p53^{NES} mutant by fusing to its C terminus a strong NES from the cAMP-dependent protein kinase inhibitor (pKI) (26). The fusion protein (p53$^{NES\text{-}pKI}$) can undergo efficient nuclear export (23) and is found in both the nucleus and the cytoplasm by immunofluorescence staining (data not shown). We then determined Lys-382 acetylation of the p53$^{NES\text{-}pKI}$ mutant. Intriguingly, even though it can undergo active nuclear export, the p53$^{NES\text{-}pKI}$ fusion protein could not be acetylated (Fig. 2*B*, *lanes 4* and *8*). This result strongly suggests that the lack of Lys-382 acetylation in the p53^{NES} mutant is not a consequence of impaired nuclear export function but is because of disruption of an unknown function(s) of p53 by the NES mutation, which is required for p53 C-terminal lysine residue acetylation.

NES Mutations Are Located within the p53 Tetramerization Domain and Disrupt p53 Oligomerization—The p53 C-terminal NES (residues 340–351) (20) overlaps with the p53 tetramerization domain (residues 325–356) (18). Studies have shown that mutations in the NES could affect both p53 nuclear export and tetramerization (20). Thus, one possible explanation for the lack of acetylation in the p53^{NES} mutant could be that the NES mutations disrupt p53 oligomerization, which is required for p53 acetylation. To determine whether p53 oligomerization is affected by the NES mutations, we tested several p53 mutants where the conserved hydrophobic residues in the NES were mutated either individually or in combination

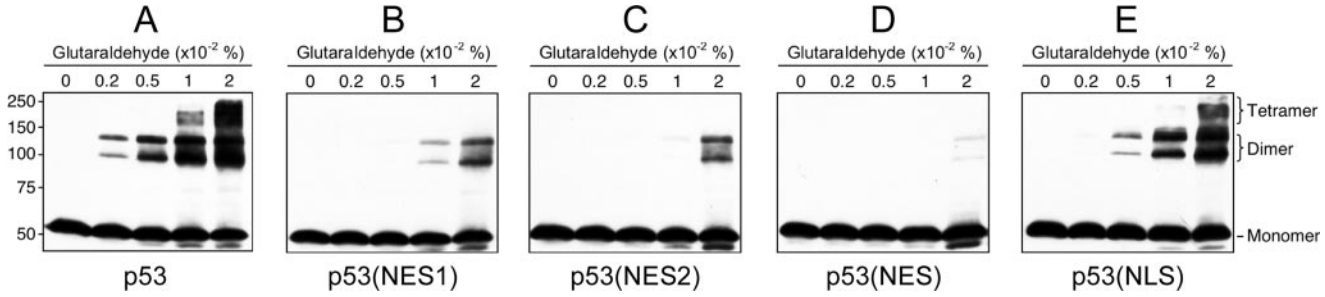

FIGURE 3. **p53 NES mutant is defective in oligomerization.** Cell lysates expressing wild-type p53 and each of the indicated p53 mutants were incubated on ice with glutaraldehyde at the indicated concentrations for 20 min. The samples were resolved on SDS-polyacrylamide gel and blotted with p53 antibody (DO-1). The molecular weight marker and the positions of monomer, dimer, and tetramer p53 are indicated.

(Fig. 1*A*). We examined the effect of the mutations on p53 oligomerization using a protein cross-linking assay. We expressed wild-type p53 and each of the p53 mutants in H1299 cells and isolated the cell lysates. The lysates were then treated with increasing amounts of glutaraldehyde to induce protein cross-linking and were separated on a denaturing SDS-polyacrylamide gel. The p53 monomers, dimers, and tetramers were analyzed by Western blotting using a p53 antibody. As illustrated in Fig. 3, we observed dimer formation with the wild-type p53 at a glutaraldehyde concentration as low as 0.002% and tetramer formation at 0.01% glutaraldehyde (Fig. 3*A*). In contrast, p53 mutants with double mutations of L348A/L350A (NES1) or M340A/L344A (NES2) formed dimers only at a high concentration of glutaraldehyde (0.02%), and no tetramers could be observed (Fig. 3, *B* and *C*). Likewise, a quadruple (L348A/L350A/M340A/L344A) p53 mutant (NES) was unable to form either dimers or tetramers at the highest concentration of glutaraldehyde tested (Fig. 3*D*). On the other hand, the p53^{NLS} mutant, which has five Lys → Ala mutations within the bipartite NLS (see Fig. 1*A*), formed dimers and tetramers with similar efficiency as did the wild-type p53 (Fig. 3*E*). These results indicate that mutations in the functional-essential hydrophobic residues of the NES affect dimer/tetramer formation of p53, which might be needed for p53 C-terminal acetylation.

Lys-382 Acetylation Does Not Occur on p53 Mutant That Is Incapable of Forming Oligomers—To investigate the relationship between p53 oligomer formation and lysine acetylation, we expressed each of the p53 NES mutants in H1299 cells and examined Lys-382 acetylation by Western blotting. Interestingly, the two dimer-capable p53^{NES1} and p53^{NES2} mutants could still be acetylated, although the intensity of acetylation of these two p53 mutants appears to be lower than that of the wild-type p53 when normalized to the total p53 protein level (Fig. 4, *lanes 3* and *4*). In contrast, the monomer-only p53^{NES} mutant was not acetylated (Fig. 4, *lane 5*). These results indicate that the p53 acetylation can take place on dimers (although maybe inefficiently) but not monomers. Because Lys-382 acetylation is believed to positively regulate p53 transcriptional activity (6), we therefore also examined whether there is a correlation between p53 acetylation and its transcriptional activity by measuring activation of endogenous p21. Our results indicated that even though the dimer-capable p53^{NES1} and p53^{NES2} mutants can be acetylated at Lys-382, their transcriptional activity was very low and was not correlated with the level of acetylation, which is consistent with the idea that a p53 tetramer has high affinity for DNA binding. Thus, p53 acetylation at the C-terminal lysine residues *per se*, without formation of tetramers, is insufficient for its activation, at least toward the p21 promoter.

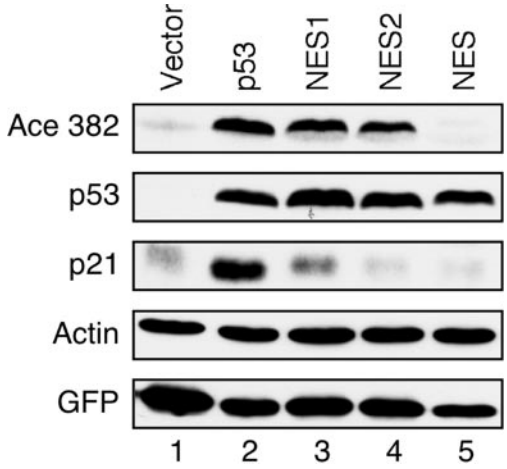

FIGURE 4. **Lys-382 acetylation does not occur on p53 mutant that is incapable of forming oligomers.** H1299 cells were transiently transfected with indicated p53 plasmids for 24 h. Cell lysates were analyzed with the indicated antibodies. GFP was used as a control for transfection efficiency.

Certain Human Cancer-derived p53 Mutations Occur in the Oligomerization Domain and Inhibit p53 Oligomerization and Acetylation—Cancer-prone germ line mutations that link to Li-Fraumeni syndrome have been shown to affect p53 tetramerization (21). We analyzed two of these familial cancer mutations, L344P and R337C, for their effect on p53 oligomerization, acetylation, and their ability to transactivate p21. Using the same protein cross-linking assay, we found that both p53^{L344P} and p53^{R337C} mutants were unable to form either dimers or tetramers at a glutaraldehyde concentration where wild-type p53 could do so efficiently (Fig. 5*A*). Furthermore, consistent with the notion that formation of oligomer is prerequisite for p53 C-terminal lysine acetylation, these mutants could not be acetylated at Lys-382 (Fig. 5*B*). Finally, to ascertain the observed Lys-382 acetylation is representative of other C-terminal lysine residue acetylations, we used a pan-Ace-p53 antibody, which recognized acetylation of four lysine sites at the p53 C terminus (Lys-372, Lys-373, Lys-381, and Lys-382) (27), and demonstrated that none of the lysine residues could be acetylated in the tetramerization mutants and a negligible low level of acetylation in the dimer mutants (Fig. 5*C*). Together,

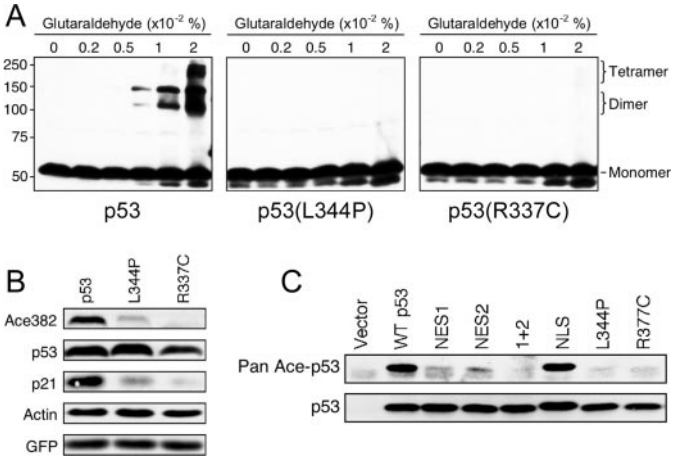

FIGURE 5. **Cancer-associated p53 mutants are defective in oligomerization, acetylation, and transactivation.** *A,* oligomer formation of wild-type p53 and two cancer-associated p53 mutants, L344P and R337C. H1299 cells were transiently transfected with indicated plasmid DNA for 24 h. Cell lysates were examined for p53 oligomerization by a protein cross-linking assay. *B,* cancer-associated p53 oligomer mutants are deficient in Lys-382 acetylation and p21 transactivation. H1299 cells were transiently transfected with plasmids expressing wild-type, L344P, or R337C mutant p53, and the cell lysates were blotted with the indicated antibodies. GFP was used as a control for transfection efficiency. *C,* p53 oligomer mutants are deficient in acetylation on multiple C-terminal lysine residues determined by a pan-Ace-p53 antibody that recognizes acetylated lysines at position Lys-372, Lys-373, Lys-381, and Lys-382 (27).

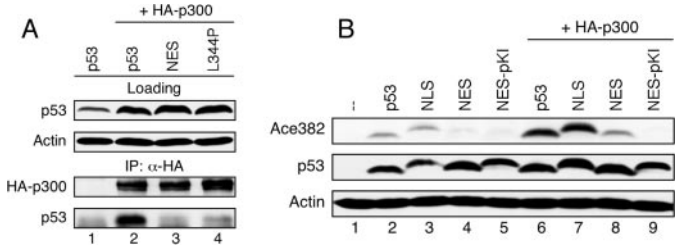

FIGURE 6. **The acetyltransferase p300 interacts with and promotes acetylation of wild-type p53, but not with mutant p53 that are defective in oligomerization.** *A,* interaction of p53 with p300 was tested by co-immunoprecipitation from the cell lysate of H1299 transiently transfected with plasmids encoding HA-p300 and various p53 as indicated. The lysates were immunoprecipitated with anti-HA antibody and blotted with the indicated antibodies. *B,* p300 promotes acetylation of p53 that are able to form tetramers. H1299 cells were transiently transfected with plasmids encoding HA-p300 and various p53 as indicated for 24 h. Cell lysates were analyzed by Western blotting with the indicated antibodies.

these results suggest that p53 oligomerization is essential prior to its C-terminal lysine acetylation.

Acetyltransferase p300 Interacts with and Promotes Acetylation of Wild-Type p53 but Not with Mutant p53 That Is Defective in Oligomerization—Our data have shown that p53 C-terminal lysine acetylation takes place on p53 tetramers (although it can also take place on p53 dimers with low efficiency), and it cannot take place on either artificially generated $p53^{NES}$ mutant or cancer-derived $p53^{L344P}$ or $p53^{R337C}$ mutants that are unable to form oligomers regardless of their subcellular localization. To elucidate the molecular basis for the requirement of p53 oligomerization for its acetylation, we tested whether oligomerization might be required for the interaction between p53 and the acetyltransferase p300. To this end, we co-transfected p300 DNA together with various p53 DNAs into H1299 cells and examined the p300-p53 interaction by immunoprecipitation-Western blotting. Consistent with previous reports (2–4), the interaction between p300 and wild-type p53 was detected in co-transfected cells (Fig. 6*A*, *lane 2*). Importantly, the interaction between p300 and each of the two monomer-only p53 mutants was reduced to nearly undetectable (Fig. 6*A*, *lanes 3* and *4*). Finally, we determined whether the p300-promoted p53 acetylation requires p53 oligomer. We co-transfected p300 DNA together with p53 DNAs into H1299 cells and examined Lys-382 acetylation by Western blotting. Co-expression of p300 promoted Lys-382 acetylation of both the wild-type p53 and the tetramer-capable $p53^{NLS}$ mutant (Fig. 6*B*, *lanes 6* and *7*) but not the tetramer-incapable $p53^{NES}$ and $p53^{NES\text{-}pKI}$ mutants (Fig. 6*B*, *lanes 8* and *9*). Thus, p300 interacts with and promotes acetylation of wild-type p53 protein but not with the p53 mutant proteins that are unable to form tetramers.

DISCUSSION

The p53 protein can be regulated with a growing list of covalently bound modifications, including phosphorylation, acetylation, ubiquitination, sumoylation, and neddylation. Post-translational modification plays critical roles in regulating p53 function through modulating its protein stability, target gene preferences, and subcellular localization. An important issue that remains incompletely understood is how these post-translational modifications of p53 are regulated. In an attempt to understand the spatial and temporal regulation of p53 acetylation, we analyzed p53 C-terminal lysine acetylation using a number of p53 mutants that localize in different subcellular compartments and are able to form tetramers, dimers, or monomers. Our study found that p53 acetylation could take place in both the nucleus and the cytoplasm. We also found that the acetylation had occurred most efficiently on tetramers.

The intracellular localization where p53 acetylation takes place has thus far not been definitively established. Wild-type p53, a primarily nuclear protein, shuttles continuously between the nucleus and the cytoplasm and therefore could theoretically be acetylated in either or both of the two compartments. Our study, using a cytoplasmic-restricted $p53^{NLS}$ mutant (23), has shown that p53 acetylation can take place in the cytoplasm very efficiently. This should not be surprising given that p300/CBP, the acetyltransferase for p53, can be found in both the nucleus and the cytoplasm (28) and was recently shown to be a shuttling protein (29). On the other hand, the possibility that the cytoplasmic localized p53 could be acetylated by a yet-unidentified acetyltransferase cannot be excluded. Evidence that p53 acetylation can take place in the nucleus is shown by our experiment in which blocking p53 nuclear export by LMB had not blocked p53 acetylation (Fig. 2*A*). The mutations introduced to the hydrophobic residues in the $p53^{NES}$ block p53 nuclear export but also inhibit p53 to form dimers and tetramers. The lack of acetylation in the $p53^{NES}$ mutant (Fig. 1) could therefore be a result of lacking oligomerization of the protein, because a nucleus-confined wild-type p53 (by treating cells with LMB) can still be acetylated. Consistent with this notion, by attaching an external NES to the $p53^{NES}$ mutant to restore its nuclear export activity, we demonstrated that the lack of acetylation in the mutant is not because of a lack of nuclear export activity

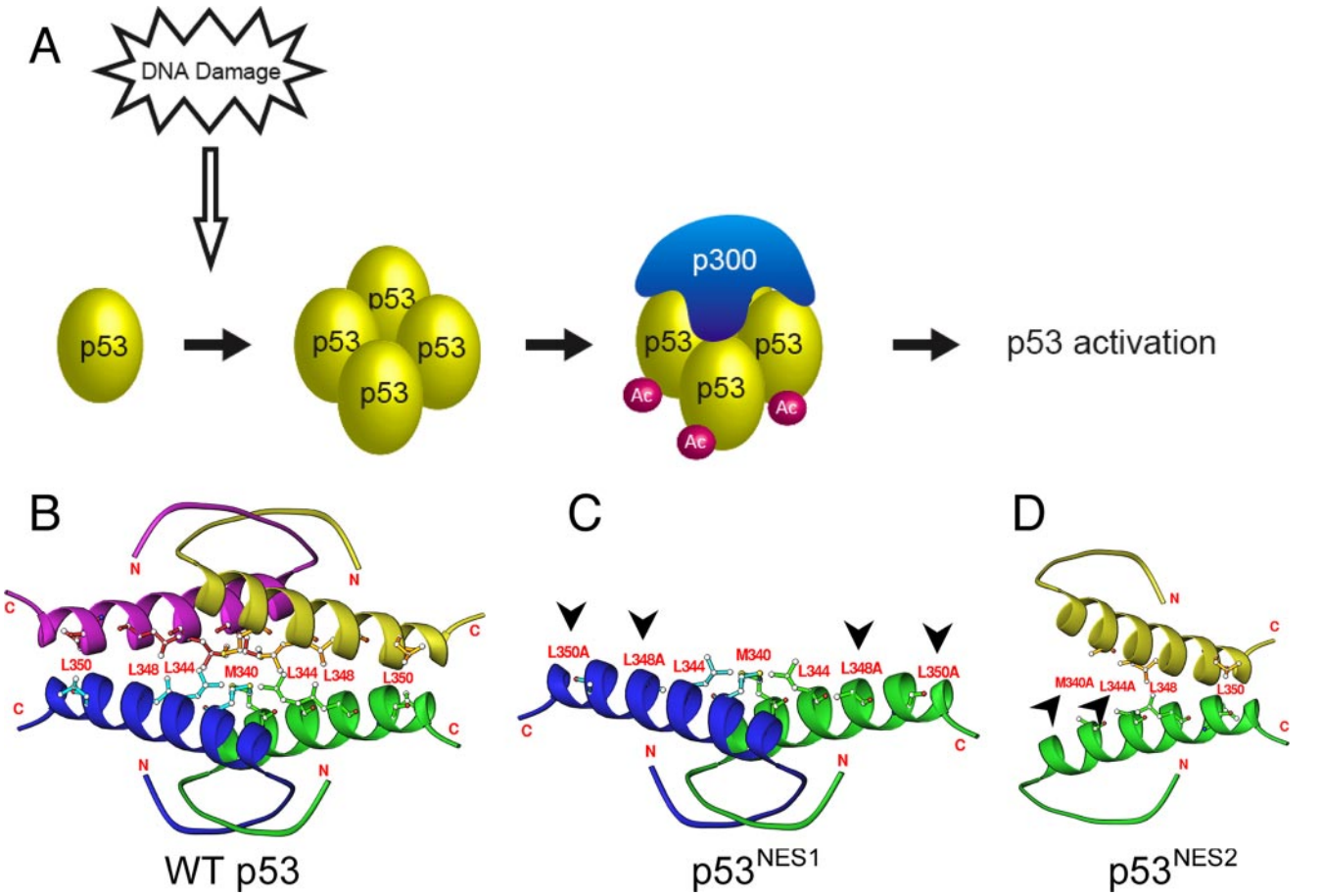

FIGURE 7. **A model for p53 activation.** *A,* model for sequential and stepwise activation of p53. DNA damage induces formation of p53 tetramer, which provides docking sites for p300, leading to p300 binding and subsequent acetylation of p53. The acetylation of p53 further tightens the p300-p53 complex, stabilizes p53, and facilitates recruitment of co-activators leading to transactivation of p53 target genes. *B–D,* ribbon diagram of p53 tetramer and dimer (constructed from the Protein Data Bank entry 1C26). The hydrophobic residues analyzed in the study are indicated in *red*. Residues tested by mutational analysis are indicated by *arrows*.

(Fig. 2*B*). Further analysis of the mutants by a protein cross-linking assay indicated that the lack of acetylation in the $p53^{NES}$ mutant is in fact a result of its inability to form oligomers (Fig. 3). By using two other p53 NES mutants ($p53^{NES1}$ and $p53^{NES2}$), whose nuclear export activity is significantly decreased (20, 23) but not completely blocked (23), together with the LMB assay, we were able to show that the p53 acetylation likely can take place in both the nucleus and the cytoplasm. This conclusion is consistent with a recent study showing that the tumor suppressor protein PTEN forms a complex with p300 in the nucleus and functions to both promote and maintain p53 acetylation (30).

Our data presented in this study support a model where p53 acetylation follows its oligomerization. We found that the acetylation cannot take place on p53 mutants that cannot form oligomers regardless of their subcellular localization. It takes place on p53 tetramers, although it can also take place on p53 dimers with low efficiency. We believe that the molecular basis for the requirement of p53 oligomerization for acetylation to occur is because the acetyltransferase p300 can interact with and promote acetylation of only p53 oligomers but not monomers (Fig. 6). Numerous putative domains in p300 are reported to interact with p53 (31–34). A recent study using NMR and fluorescence anisotropy titration has shown that four domains in p300, Taz1, Taz2, Kix, and IBiD, can each independently interact with a p53 N-terminal peptide (residues 1–57) with equal affinity (35). Therefore, it is conceivable that a p300 monomer wraps around a p53 tetramer, but not a p53 monomer or dimer, and this simultaneous interaction of four domains in p300 with each p53 molecule in a p53 tetramer further synergizes the interaction, stabilizes the p53 tetramer, and facilitates p53 acetylation. Our date implicate that p53 tetramerization precedes p300 binding and acetylation but not vise versa. This is consistent with a recent study showing that a $p53^{6KR}$ mutant, in which all five known acetylation-occurring lysine residues and one sumoylation-occurring lysine residue at the p53 C terminus are replaced by arginines, still can form a tetramer even though it cannot be acetylated (36). It would be interesting to find out if acetylation of p53 at Lys-164, which also requires p300/CBP (37), can occur on p53 monomers or still requires tetrameric p53. Based on previous and our current findings, we propose a model in which sequential and stepwise activation of p53 in response to DNA damage begins with p53 oligomerization, which provides appropriate docking sites for p300 and leads to p300 binding and subsequent acetylation of p53 C-terminal lysine residues. The acetylation, in turn, further tightens the p300-p53 complex, stabilizes p53 protein (*e.g.* through antagonizing p53 ubiquitination on the same lysine residues),

and facilitates recruitment of co-activators and transactivation of target genes (Fig. 7*A*).

Our mutational analysis has shown that double substitutions of L348A/L350A ($p53^{NES1}$) or M340A/L344A ($p53^{NES2}$) prevent p53 from forming tetramers (Fig. 3). To understand the structural basis of these mutations, we analyzed the crystal structure model of the p53 oligomerization domain based on published crystal structure information (Protein Data Bank entry 1C26). The change in p53 oligomerization status caused by these mutations can be explained on the basis of the crystal structures. The wild-type p53 C terminus forms a tetramer (Fig. 7*B*) through several hydrophobic residues (Leu-330, Ile-332, Met-340, Phe-341, Leu-344, Ala-347, Leu-348, and Leu-350) (18). The top and bottom dimers are held together by residues Ala-347, Leu-348, and Leu-350, plus some contributions from Leu-344. When both Leu-348 and Leu-350 are mutated to alanines ($p53^{NES1}$), the distances between the side chains of these residues are too far apart to form hydrophobic interactions between the dimers, and the tetramer is destroyed (Fig. 7*C*). Similarly, residues Lys-330, Ile-332, Met-340, Phe-341, Leu-344, and Leu-348 contribute to the formation of the hydrophobic core between the left and right dimers. Consequently, mutations of M340A/L344A will significantly weaken the hydrophobic interaction between the left and right dimers, also disrupting the tetramer (Fig. 7*D*). The importance of tetramerization to the tumor suppressive function of p53 was previously shown by a study in which fusion of a dimerization domain of the yeast transcription factor GCN4 to a C-terminal truncated p53 restored its transcriptional activity (38). We speculate that the fusion of the dimerization domain enabled p53 to form not only dimers but also tetramers, which is required for p53 activity. The importance of tetramerization in the tumor suppressive function of p53 was further underscored by mutations found in Li-Fraumeni syndrome patients (22) that specifically affect formation of p53 dimers and tetramers. In conclusion, our findings that the oligomerization-defective p53 mutants are also defective in acetylation and transactivation provide further explanation for a coordinated regulation of p53 function.

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